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Authors

Volkman, LE
Goldsmith, PA

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Budded *Autographa californica* NPV 64K Protein: Further Biochemical Analysis and Effects of Postimmunoprecipitation Sample Preparation Conditions

LOY E. VOLKMAN¹ AND PHYLLIS A. GOLDSMITH

Department of Entomology and Parasitology, University of California, Berkeley, California 94720

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Previously it was shown that AcV₁, a neutralizing monoclonal antibody of the *Autographa californica* nuclear polyhedrosis virus-budded phenotype reacted with a surface antigen present on infected cells during virus budding, and in the viral envelope (L. E. Volkman, P. A. Goldsmith, R. T. Hess, and P. Faulkner (1984), *Virology* 133, 354-362). Radioimmune precipitation of solubilized, [³⁵S]methionine-labeled budded virus with AcV₁ and analysis on SDS-PAGE revealed four bands consistently: one major band at 64,000 Da, and three minor bands at 127,000, 59,000, and 49,000 Da. The reason for the appearance of four bands instead of one was unclear. Data suggest that two of the bands, 49K and 59K, are aberrant, and are the products of sample preparation conditions. Further, evidence is presented that the 127K band is composed of dimers of the 64K protein, and that under nonreducing conditions, oligomers (trimers and tetramers) of 64K protein can also be detected. BVGP 64 is additionally shown to be phosphorylated and to have an isoelectric point of 3.15. The BVGP 64 epitope reactive with AcV₁ is destroyed by interaction with SDS. This could account for the lack of neutralizing activity of antiserum made to the SDS-PAGE purified BVGP 64. © 1984

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INTRODUCTION

Within the scope of our studies aimed at understanding the early events of host cell infection by *Autographa californica* nuclear polyhedrosis virus (AcNPV), and the parameters affecting specificity during those early events, we followed an experimental approach that involved the identification and characterization of the target protein of a monoclonal antibody that specifically reacted with and neutralized the budded phenotype (BV), but did not react with the occluded phenotype of the virus (Hohmann and Faulkner, 1983; Volkman *et al.*, 1984). In a previous study we determined that the target antigen was an N-linked glycoprotein that was present both in the envelope of BV and in the plasma membrane of infected cells during virus budding (Volkman *et al.*,

1984). A question generated (but which remained unanswered) by that study was why the immunoprecipitation of solubilized, [³⁵S]methionine-labeled BV yielded four bands (one major band of 64,000 Da and three minor bands of 127,000, 59,000, and 49,000 Da) instead of just one band by SDS-PAGE autoradiography. The major goal of the study reported herein was to determine the significance and/or relatedness of the four bands. By varying postimmunoprecipitation sample preparation conditions we obtained evidence that the 49K and 59K bands were breakdown products of the 64K protein (BVGP 64), and that the 127K bands were composed of dimers of BVGP 64. Under nonreducing conditions higher-molecular-weight oligomers of BVGP 64 (trimers and tetramers) could be detected in addition to dimers. We obtained evidence that BVGP 64 was an acidic phosphoprotein with an isoelectric point of 3.15. Additionally it was discovered that the BVGP

¹ Author to whom requests for reprints should be addressed.

64 epitope reactive with the neutralizing monoclonal antibody was destroyed by interaction with SDS. This effect could account for the lack of neutralizing activity of antiserum made to SDS-PAGE purified BVGP 64.

MATERIALS AND METHODS

Virus and cells. All studies were conducted with *Spodoptera frugiperda* IPLB-SF-21 cells grown at 28° in BML-TC/10 medium (Gardiner and Stockdale, 1970) with 10% fetal calf serum. The virus used was second-passage E2 variant of AcNPV BV (Smith and Summers, 1978).

Isotopic labeling. Labeling of AcNPV BV with [³⁵S]methionine was as described previously (Volkman *et al.*, 1984). Labeling with ³²PO₄ was basically as described by Maruniak and Summers (1981), but with a few changes. A nearly confluent monolayer of IPLB-SF-21 cells in a 75-ml culture flask was infected at 28° with BV at an m.o.i. of 10. After a 2-hr adsorption period the inoculum was removed and the cells were rinsed with phosphate-labeling medium—phosphate-free BML-TC/10 medium containing 2% dialyzed fetal calf serum and 10 mM HEPES, pH 6.1. After the rinse, 8 ml labeling medium was added back to the flask, followed by 0.5 mCi H₃³²PO₄ in 5 μ l. The cells were incubated at 28° and rocked gently on a Belco rocker platform until BV was harvested at 27 hr postinfection (p.i.).

Immunoprecipitation, SDS-PAGE, and autoradiography. The basic procedure followed was as before (Volkman *et al.*, 1984) but included a few standard changes, and some variations for specific experiments. The standard changes were that solubilized virus was centrifuged at 100,000 *g* (instead of 50,000 *g*) for 1 hr at 4° to pellet any insoluble proteins and structures before immunoprecipitation, and, unless specifically stated differently, immunoprecipitated samples were boiled 3 min only before being loaded onto the gel. The gels used in SDS-PAGE varied in acrylamide concentration from 7.5 to 10%. Concentrations of particular gels are indicated in the figure legends. Concentrations of presolubilized BV used per well

ranged from 9 to 75 μ g, depending on the experiment. Immunoprecipitated samples were resuspended in sample buffer composed of 0.125 *M* Tris-Cl, pH 6.8, 20% glycerol, 2% SDS, 3% 2-mercaptoethanol (2-ME), and 0.01% bromophenol blue. In certain indicated experiments, 0.075 *M* iodoacetamide (IA) replaced the 2-ME in the sample buffer, and/or the pH was adjusted to 2.3 with HCl. Exposure times of the gels to the X-ray film varied from 3 to 14 days.

Isoelectric focusing. Isoelectric focusing was carried out on 0.45 ml solubilized BV per experiment (about 400 μ g before solubilization) from four 75-ml flasks of infected, [³⁵S]methionine-labeled cells. The virus was harvested at 26–28 hr p.i. The procedure of Hagland (1967) was followed using an ampholine buffer, pH 2.5–6.0, and a sucrose-ampholine gradient. The protein was focused in an LKB 8101 column at 300 V and 4° for 48 hr. The column was drained and 2.7-ml fractions were collected. The pH of each fraction was measured, then all fractions were adjusted to pH 7.4 by dialysis for 48 hr with two changes of 0.01 *M* Tris-HCl buffer. A 0.1-ml sample from each fraction was suspended in 0.4 ml Aquaflo and counted in a Packard scintillation counter. For radioimmune precipitation, sample volumes were reduced to 1 ml using Amicon centriflo ultrafiltration membrane cones with a molecular-weight cutoff of 25,000 (CF 25), and incubated with AcV₁ charged Protein A-Sepharose CL-4B beads overnight at 4° on a rocker platform.

Reduction of high-molecular-weight bands. Gel slices containing the individual 229K, 172K, 129K, and 64K bands from a [³⁵S]methionine-labeled, IA-treated sample were rehydrated by soaking in 40% methanol for two 30-min periods on a rocker platform, followed by a final soak in pH 6.8 sample buffer with 2-ME. Each gel fragment was boiled 3 min in sample buffer (pH 6.8 with 2-ME) and then carefully pushed to the bottom of a well of a 7.5% gel. The residual sample buffer was then layered on top of the respective fragments and the samples were electrophoresed and analyzed as usual.

RESULTS

Effects of postimmunoprecipitation sample preparation conditions. Previous experiments that had yielded four bands upon radioimmune precipitation with AcV₁ were done under conditions of boiling the precipitated antigen for 10 min in sample buffer, pH 6.8, containing 2% SDS and 3% 2-ME before being loaded onto 10 or 12% SDS-PAGE gels (Volkman *et al.*, 1984). We examined the effect of varying the chemical environment and the time of boiling on the number and intensity of the bands obtained. Samples were boiled for 3 min or longer in pH 6.8 sample buffer with or without 3% 2-ME, or were heated to 100° or incubated at room temperature for 3 min in pH 2.3 sample buffer with or without 3% 2-ME. The results of these treatments are shown in Fig. 1. The 64K band was prominent in all cases, and the 127K band was visible, although faintly so, in all conditions tested except for boiling in pH 2.3 sample buffer with 3% 2-ME (Fig. 1, lane C). In contrast, the appearance of the 59K and 49K bands was highly dependent upon the sample preparation conditions, with the appearance of both being favored by boiling in sample buffer of pH 2.3 (Fig. 1, lanes C and D). Antigens released by the acid environment of this same sample buffer without being boiled contained no detectable 59K band and only traces of the 49K protein (lanes E and F). In sample buffer of pH 6.8, samples boiled in the presence of 2-ME contained a heavier 49K band than those boiled in the absence of 2-ME (compare lanes A and B). Extended boiling times also favored the appearance of the 49K protein (compare lanes G, H, and I). The appearance of various peptides less than 49K also depended on sample preparation conditions, as is evident in (A) through (F).

Because of the intensity of the peroxidase antiperoxidase staining reaction associated with the terminal regions of the virus containing peplomers reported previously (Volkman *et al.*, 1984), and the possibility that the 127K band could be a dimer of the 64K protein, we wanted to

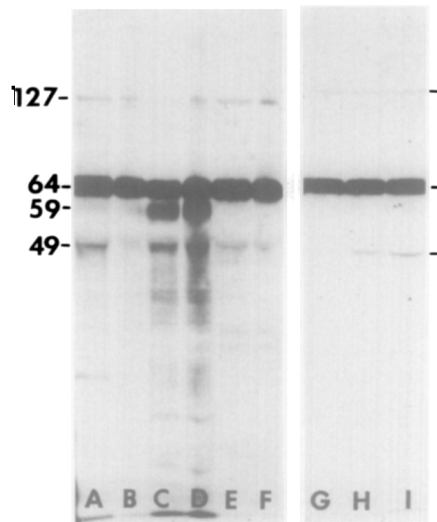


FIG. 1. Effects of postimmunoprecipitation sample preparation conditions. AcV₁ immunoprecipitated, [³⁵S]methionine-labeled, solubilized BV protein samples were treated various ways before SDS-PAGE on 10% gels: resuspended in pH 6.8 sample buffer with 3% 2-ME (A) or without 2-ME (B) and boiled 3 min; resuspended in pH 2.3 sample buffer with 3% 2-ME (C, E) or without 2-ME (D, F), and boiled 3 min (C, D), or not boiled (E, F); resuspended in pH 6.8 sample buffer with 3% 2-ME and boiled 3 min (G), 5 min (H), or 10 min (I). In (A)–(F) approximately 20 µg presolubilized BV per well and 10-day exposure period were used. In (G)–(I), 12.5 µg presolubilized BV and a 5 day-exposure period were used.

determine the effect of nonreducing conditions and prevention of possible thiol-disulfide interchange leading to disulfide bond disruption (Victoria *et al.*, 1977) on the molecular weights of the bands obtained by immunoprecipitation with AcV₁. To accomplish this we added alkylating agent IA (0.075 M) to pH 6.8 sample buffer in place of the standard 2-ME, and observed bands with apparent molecular weights of 64K, 129K, 172K, and 229K upon SDS-PAGE analysis (Fig. 2, lane C). Controls of nonimmunoprecipitated solubilized virus 100,000 g supernate were included to confirm that the higher-molecular-weight bands were not aggregates somehow caused by IA, but were present in the 100,000 g supernatant sample fraction (Fig. 2, lane B). Both the nonimmu-

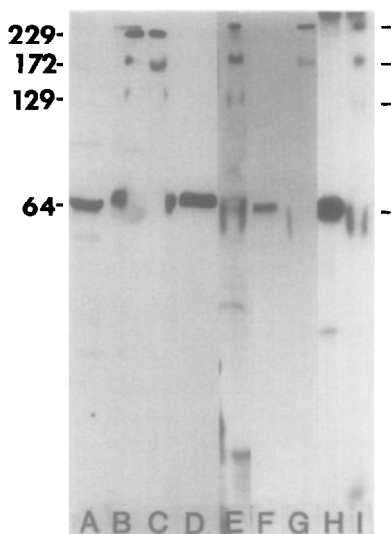


FIG. 2. Effect of nonreducing conditions and sulfhydryl alkylation. [35 S]methionine-labeled BV was solubilized as usual (A, B, C, D), or in the presence of 0.075 *M* IA (E, F, G) and analyzed without immunoprecipitation, exposure to 2-ME, or boiling (B, E), or with boiling 3 min in sample buffer with 2-ME (A). AcV₁ immunoprecipitated samples, labeled with either [35 S]methionine (C, D, F, G) or 32 PO₄ (H, I) were boiled 3 min in sample buffer containing 3% 2-ME (D, F, H) or 0.075 *M* IA (C, G, I) before SDS-PAGE. In (A)–(G), approximately 9.1 μ g presolubilized BV was used per well, and in (H)–(I), 25 μ g per well. Exposure time was 3 days for all. (A)–(D) is a 10% acrylamide gel; (E)–(I) is a 7.5% acrylamide gel.

noprecipitated and the IA-treated higher-molecular-weight protein bands were sensitive to 2-ME and disappeared in its presence with no other heavily labeled bands appearing to take their place other than the 64K band (Fig. 2, lanes A and F). An attempt was made to determine whether these higher-molecular-weight aggregates were intact in the presolubilized virus or if they aggregated after solubilization by pretreating labeled virus with IA for 30 min at room temperature, and solubilizing in its presence for 2 hr at 4° before immunoprecipitation. The results of these experiments indicate that the higher-molecular-weight protein aggregates were present in the intact virus (Fig. 2, lanes E and G).

Phosphate labeling of BVGP 64. Budded

virus grown in medium containing 32 PO₄ was solubilized and the standard immunoprecipitation procedure with AcV₁ was performed. The antigen was released from antibody by boiling for 3 min in pH 6.8 sample buffer containing either 3% 2-ME or 0.075 *M* IA. The results were a prominent 64K band in the 2-ME lane and the characteristic 64K, 129K, 172K, and 229K bands in the IA lane (Fig. 2, lanes H and I).

Reduction of 129K, 172K, and 229K complexes to 64K. To investigate further the relationship between the higher-molecular-weight complexes and BVGP 64, the 129K, 172K, and 229K bands resulting from a sample treated with IA were excised from a gel, soaked in methanol, and rehydrated in buffer with 3% 2-ME before being boiled 3 min and resubjected to SDS-PAGE. The results were that in the presence of 2-ME, all the higher-molecular-weight complexes were reduced to 64K bands (Fig. 3, lanes C, D, and E). The 64K protein from the IA-treated sample re-banded primarily at 64K upon reanalysis by PAGE in the presence of 2-ME, with slight degradation into lower-molecular-weight peptides (lane F). Much more extensive degradation occurred when the 64K protein from a sample previously treated with 2-ME was reanalyzed (G).

Comparison of BVGP 64 antiserum and AcV₁'s recognition of BVGP 64. It was reported previously that antiserum made to AcNPV BV's SDS-PAGE purified 64K protein (BVGP 64) did not neutralize AcNPV BV (Volkman *et al.*, 1984), even though BVGP 64 was clearly the major target of neutralizing monoclonal antibody AcV₁. This observation and others led to the speculation that the integrity of the target epitope of the neutralizing AcV₁ might be dependent upon the association of at least two proteins or peptide chains, with the 64K protein being at least one of the components. Alternatively, it was reasoned, the neutralizing epitope was destroyed by the Laemmli gel sample preparation conditions. The evidence presented in the previous sections, suggesting that BVGP 64 could be present as multimers in the virion (perhaps forming the

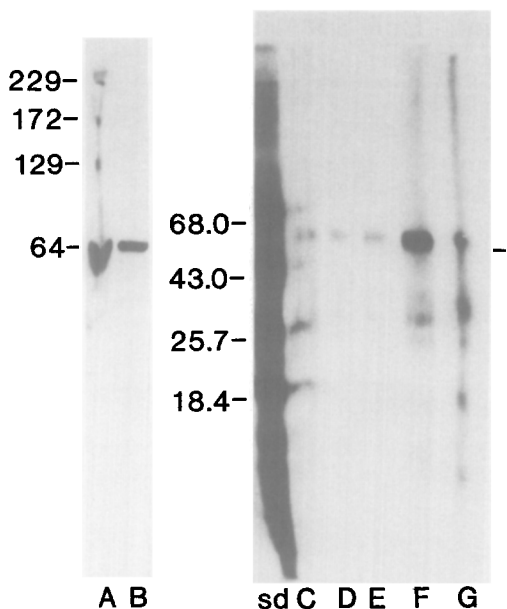


FIG. 3. Reduction of 229K, 172K, and 129K complexes to 64K. AcV₁ immunoprecipitated sample of [³⁵S]methionine-labeled solubilized BV boiled 3 min in pH 6.8 sample buffer containing 0.075 M IA (A) or 3% 2-ME (B) before SDS-PAGE on a 7.5% gel. Bands excised from gel pictured in (A), boiled in pH 6.8 sample buffer containing 3% 2-ME, and resubjected to SDS-PAGE on a 7.5% gel: (C) 229K, (D) 172K, (E) 129K, and (F) 64K. Lane (G) is the band excised from (B) and resubjected to SDS-PAGE. Molecular-weight standards $\times 10^{-3}$ (sd).

peplomers) presented the further possibility that the multimeric form of BVGP 64 might be the functional target of neutralizing AcV₁. It was of interest, therefore, to determine whether the antiserum to SDS-PAGE purified BVGP 64 could recognize multimers of BVGP 64, whether AcV₁ could recognize reduced and alkylated BVGP 64, and what component of the Laemmli protein disruption treatment destroyed the AcV₁ recognition (neutralizing) epitope. The experiment designed to answer these questions was simply to preboil the 100,000 *g* supernatant fraction of [³⁵S]methionine-labeled, solubilized BV for 3 min with 0.075 M IA, 1% SDS, or 3% 2-ME followed by 0.075 M IA, and immunoprecipitate using either AcV₁ or antiserum to BVGP 64. The immunoprecipitated antigens were then boiled for 3 min in pH 6.8 sample buffer containing

either 3% 2-ME or 0.075 M IA prior to SDS-PAGE. The results indicated that the BVGP 64 antiserum recognized the multimers of BVGP 64 (Fig. 4A, lane D), and AcV₁ recognized reduced and alkylated BVGP 64 (Fig. 4B, lane A). Boiling 3 min in IA did not destroy the AcV₁ reactive epitope, but boiling 3 min in 1% SDS did (Fig. 4A, lanes F, I, and J). In contrast, a 3-min boil in 1% SDS appeared to enhance recognition by the antiserum to BVGP 64 (Fig. 4A, compare lanes K and L with C and D). It was also noted that boiling in IA prior to immunoprecipitation led to some apparent aggregate formation, even if the sample was prereduced by boiling in 2-ME, because labeled material appeared at the origin of the gels containing these samples (Fig. 4A, lanes E, F, G, H, and Fig. 4B, lanes A and B).

Isoelectric focusing of BVGP 64. The 100,000 *g* supernatant fraction of solubilized [³⁵S]methionine-labeled BV was subjected to isoelectric focusing on a sucrose-ampholine gradient, and collected fractions were tested for pH, counts per minute content, and reactivity with AcV₁ by immunoprecipitation. A peak of radioactivity occurred at pH 3.15, ranging from pH 3.07 to 3.28 (Fig. 5). The peak fractions contained a 64K protein that was immunoprecipitated by AcV₁ (not shown).

DISCUSSION

The major problem addressed initially in this study was to assess the significance of the four bands (127K, 64K, 59K, and 49K) obtained by immunoprecipitation of solubilized BV with AcV₁ (Volkman *et al.*, 1984). It was determined that the 49K and 59K bands were generated by postimmunoprecipitation sample preparation conditions such as heating to 100° (especially at pH 2.3), and that exposure to 2-ME exaggerated the effects of heating. That the 64K protein can be degraded by the combined effects of 2-ME and boiling was confirmed by the observation of breakdown products by a second PAGE analysis of PAGE-purified 64K protein (Fig. 3). Boiling in IA, and boiling at pH 2.3 led to the generation of numerous other bands

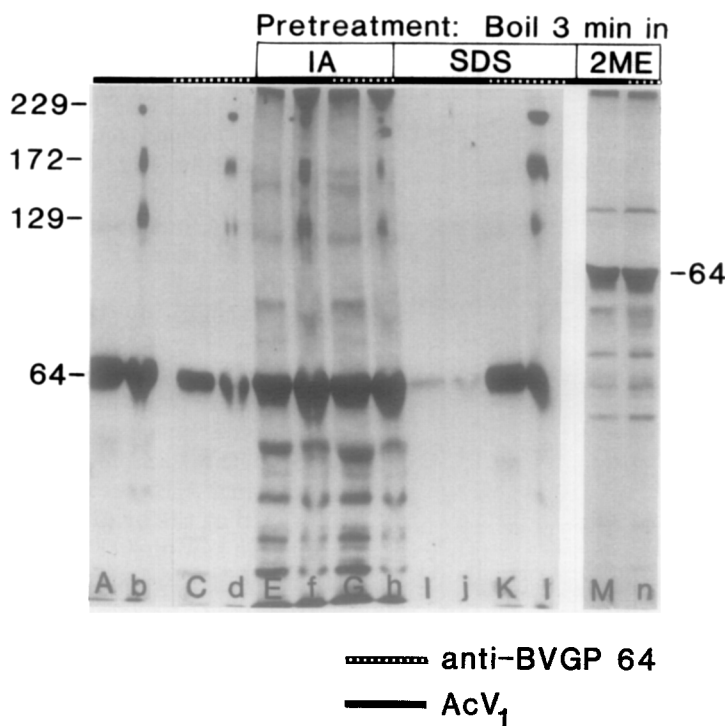


FIG. 4. Effect of pre- and postreaction sample conditions on immunoprecipitation by AcV₁ and antiserum to BVGP 64. [³⁵S]methionine-labeled, solubilized BV 100,000 *g* supernate was either not boiled (A, b, C, d) or boiled 3 min with 0.075 *M* IA (E, f, G, h), 1% SDS (I, j, K, l), or 3% 2-ME followed by a 3 min boil with 0.075 *M* IA (M, n) before immunoprecipitation with AcV₁ or BVGP 64 antiserum. Immunoprecipitated samples in lanes labeled with capital letters were boiled 3 min in pH 6.8 sample buffer containing 3% 2-ME before SDS-PAGE. Samples in lanes labeled with lower case letters were boiled in pH 6.8 sample buffer containing 0.075 *M* IA before SDS-PAGE. Gel pictured in (A)–(l) is 7.5% acrylamide; (M)–(n) is 10% acrylamide.

in addition to the four first observed (Figs. 1 and 4). These types of effects, that is, the generation of anomalous bands on SDS-PAGE gels by heating to 100°, especially in the presence of 2-ME, have been described before for a coronavirus envelope protein (Sturman, 1977). The significance of these observed effects with regard to baculovirology in general is that care must be taken in evaluating molecular weights and numbers of proteins observed by SDS-PAGE, both with and without subsequent autoradiography or Western blotting. (The effects, of course, are exaggerated with the more sensitive detection techniques.) It is quite possible that many of the observed bands, even though they are consistently obtained, may be due to protein degradation or aggregation,

or both. Effects such as these could account for the reaction of multiple bands with individual monoclonal antibodies in Western blots of viral structural proteins, such as described by Hohmann and Faulkner (1983).

The consistent appearance of the major 64K and the minor 127K bands led to the hypothesis that the 127K band was composed of dimers of the 64K protein, perhaps connected by disulfide bonds. Removal of 2-ME from the sample buffer did not lead to the appearance of a heavier 127K band (Fig. 1, lane B) but it has been reported that disulfide bonds can be rapidly broken by a thiol-disulfide interchange process if samples are heated to 100° in SDS solutions (Victoria *et al.*, 1977). It was further reported that this

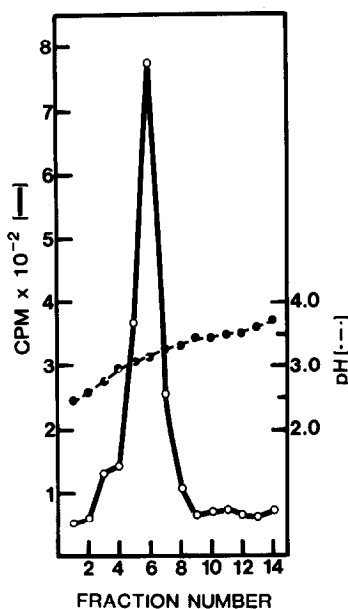


FIG. 5. Isoelectric focusing of 100,000 *g* supernate of [³⁵S]methionine-labeled, solubilized BV.

process can be inhibited and disulfide bonds preserved if IA is present during the heat treatment (Victoria *et al.*, 1977). This information led to the experiments wherein IA was substituted for 2-ME in the sample buffer. When immunoprecipitated samples were prepared for SDS-PAGE by boiling in sample buffer containing IA, bands of molecular weights 229K, 172K, and 129K were obtained in addition to the 64K band. That these bands were composed of oligomers of 64K was demonstrated by their subsequent reduction, individually, to 64K (Fig. 3). It is probable that these bands represented dimers (129K), trimers (172K), and tetramers (229K), and that the molecular weights reported here are inaccurate. It is known that accurate molecular weight values are exceedingly difficult to obtain for glycoproteins by SDS-PAGE, because of the effect of the carbohydrate. For example, molecular-weight estimates vary with the percent acrylamide used in the gel (Scheid and Choppin, 1977).

An attempt was made to assess whether 64K oligomers were present in the intact virions (and did not form by aggregation during solubilization) by solubilizing BV

both in the presence and absence of IA, and analyzing a sample of nonimmunoprecipitated 100,000 *g* supernate. It has been reported that IA prevents aggregation of immunoglobulins (Virella and Parkhouse, 1973). The results showed that oligomers were present in the 100,000 *g* supernate of both samples (Fig. 2, lanes B and E). These results supported the interpretation that the oligomers were present in the intact virus, and were not generated by postimmunoprecipitation boiling in sample buffer with IA, or by aggregation during solubilization. The results further indicated that BVGP 64 and multimers of it were the major labeled species present in the 100,000 *g* supernate.

The probable presence of oligomers of BVGP 64 in the intact virus led to the hypothesis that they may compose the peplomers of BV, especially in view of the intense AcV₁ mediated immunological staining previously noted in the peplomer region of the virus (Volkman *et al.*, 1984). It was reasoned that if this in fact were the case, then maybe the oligomers (peplomers) are the functional targets of the neutralizing antibody, which might be the reason for the differing abilities of AcV₁ and BVGP 64 antiserum to neutralize BV. It was therefore of interest to determine whether neutralizing AcV₁ could recognize monomeric (reduced and alkylated) BVGP 64, and conversely, whether nonneutralizing antiserum to BVGP 64 could recognize the oligomers. It was found that both AcV₁ and BVGP 64 antiserum could recognize both monomers and oligomers, so the difference in observed neutralizing activity was not due to recognition (or lack of recognition) of complexes of BVGP 64. The explanation for the difference in neutralizing ability of the two came when it was observed that boiling BVGP 64 in 1% SDS destroyed its reactivity with AcV₁, but enhanced its reactivity with BVGP 64 antiserum (Fig. 4, lanes I, J, K, and L). The epitope important for neutralizing activity was destroyed by interaction with SDS.

In a study of AcNPV phosphoproteins, Maruniak and Summers (1981) determined that the 64,000-molecular-weight struc-

tural protein of extracellular virus was phosphorylated. We confirmed this finding and additionally determined that it was highly acidic, as is characteristic for phosphoproteins, with an isoelectric point of 3.15.

In summary, BVGP 64 is an abundant, acidic, phosphoglycoprotein found in the envelope of AcNPV BV. BVGP 64 is important in the infectivity of AcNPV BV as it is the target of neutralizing antibody AcV₁. Its biological function, however, is still unknown at this time. It is likely that BVGP 64 composes the peplomers seen projecting from BV envelopes, though this by no means is firmly established. The occluded phenotype of AcNPV also has a 64K structural phosphoprotein (Maruniak and Summers, 1981). It is doubtful that this protein is the same as BVGP 64, however, because it does not appear to be a glycoprotein (Stiles and Wood, 1983), and it does not react with any of four monoclonal antibodies (AcV₁, AcV₅, AcV₆, and AcV₁₄) reactive with BVGP 64 (Hohmann and Faulkner, 1983). This point must be examined more thoroughly, however, before any firm conclusions are drawn.

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